throughout the titration, independent of the nanosecond fluorescence changes of W43. This leads us to believe that the W43 rise is not reporting simply the dynamics of the first hydration layer, but instead upon longer range solvation.
To rationalize these observations, three sets of 300 2-ns MD+QM simulations were carried out for different levels of protonation of glutamate and aspartate residues. During simulations Trp is switched from ground charges to 1La charges, and the average fluorescence wavelength was computed vs. time. Early results are consistent with the observation of a ~30 ps FDSS component, independent of pH. The protonated carboxyl group of Glu27 is solvated and intermittently comes in close contact with the Trp ring, strongly suggesting that electron transfer to this group causes ns lifetime shortening at lower pH. The trajectories also show that Lys31 and Glu27 lie salt-bridged above the Trp pyrrole ring at high pH. The blue shifting of the emission at lower pH is consistent with the Lys31 positive charge over the pyrrole ring at low pH.

1767-Pos Board B659
Thioamides as Fluorescence-Quenching Probes
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Fluorescence-quenching probes can be used to study the dynamics, folding, and aggregation of appropriately labeled protein systems. Valuable structural information can be extracted from distance-dependent quenching experiments; however, the structural resolution is often limited by the large size of the chromophore labels typically required for this technique. One solution is to use smaller quenchers that do not significantly perturb native protein structure. We have shown that backbone thioamides, which are single-atom substitutions of a peptide bond, quench a variety of fluorophores in a distance-dependent fashion through Förster resonance energy transfer (FRET) or photoinduced electron transfer (PET) mechanisms. Since thioamide analogs of the natural amino acids can be incorporated at almost any position in a protein sequence, they can be paired with bulkier fluorophores that perhaps can only be tolerated in a few positions in a protein. We have used this method to monitor the binding of thioamide-containing peptides to calmodulin, the unfolding of the villin headpiece subdomain, aggregation of α-synuclein, and protease activity, among other applications.

1768-Pos Board B660
Laurdan Spectral Phasor Detects Membrane Micro-Heterogeneity and Lipid Domains in Live Cells
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The fluorescent probe Laurdan is sensitive to membrane lateral packing and lipid order. Laurdan is commonly used in model and biological membranes to report on different lipid phases. Here we describe a novel approach to detect Laurdan spectral changes due to membrane packing in vivo: the Laurdan spectral phasor. By Fourier transformation of Laurdan's spectrum into a spectral phasor we obtain two coordinates for each pixel of an image. The linear combination of two spectral components always falls in the line joining the phasors representing the two components, which allows quantification of the relative contribution of the different lipid phases. If, however, there is a different environment for Laurdan in a pixel, the phasor of that pixel cannot be represented by the linear combination of the phasor of two components. Therefore, nonlinear environment interactions can be immediately detected. We use this approach to perform a comprehensive analysis of membrane heterogeneity in NIH3T3 live cells. We take advantage of a greater sensitivity, compared to conventional techniques, and we are able to detect highly packed micro-domains and to monitor changes in membrane packing due to acute and chronic cholesterol manipulation in vivo. Work supported in part by NIH-P41 P41-RRO3155, 8P41GM103540 and P50-GM076516.

1769-Pos Board B661
New Solution of Eliminating the Inner Filter Effect in Fluorescent Measurements
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A method of correction and normalization of the experimentally recorded fluorescence intensity allowing to exclude the influence of the so called inner filter and to select an informative component that is the product of absorbance and fluorescence quantum yield of fluorophore in the under test solution is proposed: $F_0 = F/kW = A_{fl}\, q_{fl}$. The value of the correction factor $W = (1\text{-}10^{-A\Sigma})/A_\Sigma$ is determined solely by the absorbance of the solution A_Σ, and does not depend on the relative contribution to the absorption of fluorescing and non-fluorescing components of the solution. The normalization factor k can be determined from the measurement (in the same conditions in which the fluorescence intensity of the sample is measured) of reference fluorescence, i.e. the solution of the substance with a known fluorescence quantum yield.
It is shown that the proposed method of fluorescence intensity correction and normalization allows determining undistorted fluorescence excitation spectra of solutions with high optical density and 3D-fluorescence spectra, correcting Stern-Volmer dependences if a quencher absorbs at the wavelength of the exciting light. Registration of the fluorescent dye-receptor system fluorescence intensity on the concentration of the dye allows determining the binding constant and the number of binding sites of the dye to the receptor if all of the bound dye molecules, which are responsible for fluorescence, belong to a single binding mode. The complete information about the parameters of the dye-receptor interaction, as well as absorption spectra of dye bound to receptor can be obtained by spectrophotometry using a sample solution and reference solution prepared by an equilibrium microdialysis. Registration of the fluorescence intensity of these solutions also allows determining the fluorescence quantum yield of dye molecules bound to the sites belonging to different binding modes.

1770-Pos Board B662
Fast and Reliable Measurement of Photoluminescence Quantum Yields for the Development of Fluorescent Probes
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Besides molar absorption coefficient and excited state lifetime, the photoluminescence quantum yield is the key parameter to be optimized for highly efficient and bright fluorescent probes. An easy and reliable way to determine the quantum yield is therefore the prerequisite for modern fluorescent probe development.
Photoluminescence quantum yield measurements are typically done by comparing the emission intensity of the target compound with a standard of known quantum yield, under identical measurement conditions. This method is well established and precise, but also time consuming. In addition target compound and reference have to have similar absorption and emission spectra. For those cases where a suitable standard is not available; when the measurement of the absorption is cumbersome, when the determination speed is an issue, or generally for scattering samples, the use of an integrating sphere to measure the absolute photoluminescence quantum yield is mandatory.
Here we show that absolute photoluminescence quantum yield measurements of solutions as well as solid samples can be easily realized using a simple integrating sphere accessory for a conventional fluorescence lifetime spectrometer. This allows to acquire all relevant fluorescence characteristics with one instrument, therefore streamlining the characterization workflow and keeping all calibration schemes simple.
For the validation of our new assembly, selected quantum yield standards have been measured and the data were compared to literature data previously determined with a calibrated spectrofluorometer and two calibrated integrating sphere setups [1]. Procedures for the determination of the instrument's spectral sensitivity and attainable precision of the results will be discussed.
[1] Würth, C.; Pauli, J.; Lochmann, C.; Spieles, M.; Resch-Genger, U. Anal. Chem. 2012, 84, 1345-1352.

1771-Pos Board B663
Fluorescence Lifetime Microplate Reader for Structural Biology: High Performance and High-Throughput in the Same Tool
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Microplate readers are widely considered as designed and intended for high-throughput (HT) applications such as drug discovery, whereas ''research-grade'' (RG) fluorescence spectrometers are the tools of choice when quality and diversity of data are the prime considerations. However, a definite trend of the past decade is the commercial availability of microplate readers that come equipped with emission and excitation monochromators (even double monochromators) very similar to those found on research-grade spectrometers. The flashlamp sources used in most microplate readers are very similar or even identical in design to the flashlamp sources of the research-grade spectrometers. Microplate readers that employ fluorescence lifetime as a primary readout represent another possible form of convergence between the high-throughput assay and research. An interesting question is to what degree the biggest

difference between these two worlds is now simply the sample format, i.e., cuvette vs. microplate. It seems reasonable to assume that the cuvette with its straightforward implementation of right angle excitation-emission geometry offers significant advantages over the epi-illumination geometry and uncontained sample imposed by a microplate when it comes to data quality. We have implemented a prototype microplate reader equipped with a variety of pulsed laser sources for measurement of fluorescence spectra, fluorescence lifetimes, and anisotropy. The subject of this poster is benchmarking its performance relative to cuvette format. The plate reader employs direct waveform recording as an alternative to TCSPC; studies to compare the speed, accuracy, and precision of the two lifetime approaches are presented along with several examples of titration curves for rapid determination of binding affinities via time-resolved FRET.

1772-Pos Board B664

Fluorescence Quenching of Tryptophan and Tryptophanyl Dipeptides in Solution

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We report measurements of fluorescence quantum yields of tryptophan, tryptophanylaspartate and tryptophanylarginine in several solvents as well as in aqueous solutions over a wide range of pH. We aim to test a computational model developed by Callis and coworkers [Vivian, J.T. and Callis, P.R. Chem. Phys. Lett. **2002**, *369*, 409] of fluorescence quantum yield, which postulates that quenching in tryptophan arises from energy loss due to an electron transfer from the aromatic system of tryptophan to one of the amides in the protein backbone. Since the electron transfer state is expected to be high in energy, normally this would not be a possible outcome, but because of its large dipole, such a state should be more accessible in polar solvents. In addition, conditions of low (high) pH, which result in a net positive (negative) charge for the backbone should result in an increase (decrease) of electron transfer rates and low (high) quantum yields. The observed results confirm the predictions of the model.

1773-Pos Board B665

Multiscale Diffusion of Single Molecules in Biomimetic Crowding

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Molecular crowding in living cells is believed to influence diffusion processes, intermolecular interactions, protein folding, and intracellular transport. Here, we have investigated crowding effects on the rotational and translation diffusion of Rhodamine green (RhG) and enhanced green fluorescent proteins (EGFP); as compared with homogeneous solvents (buffer and glycerol). Time-resolved fluorescence anisotropy (picoseconds - nanoseconds) and fluorescence correlation spectroscopy (microseconds - seconds) were used to elucidate the effect of non-specific binding on of RhG and EGFP diffusion in synthetic (Ficoll-70, Ficoll-400) and proteins (bovine serum albumin, BSA, and ovalbumin) biomimetic crowding. Using Stocks-Einstein model, the measured rotational-to-translational diffusion coefficient ratios of RhG and EGFP indicate that the non-specific binding and deviation from Brownian diffusion depend on the type of crowding agents. These results provide new insights into crowding effects on diffusion and nonspecific binding of fluorophores on multiple scales of time and concentration.

1774-Pos Board B666

Characterization of Fluorescent Base Analogs to Study DNA Base Flipping at the Single Molecule Level

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Chemical damage to DNA bases can result in mutations, block replication, and lead to cancer. It has been suggested that the phenomenon of base flipping take place by some enzymes during the repair of DNA damages. However, it still remains to be answered if the enzyme ''pushes'' the nucleotide out of the helix (active mechanism) or if the enzyme binds to a provisional flipped base (passive mechanism). Single molecule fluorescence has demonstrated to be a powerful technique to determine the formation of one or more intermediates, and to study the kinetics of the processes from the instant before an enzyme interact with the DNA until the release of the enzymatic product, one molecule at a time. Therefore, in order to optimize and maximize the repair of damaged DNA, new single molecule approaches to fully assess the kinetic mechanism of the base flipping process are needed.

In previous work, the adenine fluorescent base analog 2-aminopurine (2AP) has been extensively used to study base flipping in ensemble average experiments. In addition, a novel 2AP single molecule approach was recently developed.[1] In order to generate single molecule fluorescence assays to probe base flipping in different DNA-enzyme complexes, we need to study fluorescent base analogs (FBA) for all the natural bases. Several FBA molecules have been synthesized during the last four decades and we have selected one FBA molecule for each DNA base to probe base flipping. We have characterized the fluorescent properties of different FBA-substituted DNA molecules that mimic the different states proposed for the base flipping process.

[1]Alemán, E.A., Patrick, E., de Silva, C., Musier-Forsyth, K. & Rueda, D. Single-molecule dynamics with fluorescent nucleotide analogues. *In preparationto be submitted*

1775-Pos Board B667

Temporal Dynamics and FRET Restrained Modeling of an "Invisible" Excited State of T4 Lysozyme

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Conformational fluctuations play a central role in enzyme catalysis. However, extracting a 4D view, i.e. structural changes over time, has represented a big challenge in molecular biophysics. For example, in most cases not all conformers of proteins are visible using the standard structural techniques such as X-ray crystallography or nuclear magnetic resonance. In this work, we present our approach using a fluorescence spectroscopic toolbox to resolve three different conformers of the bacteriophage T4 lysozyme (T4L) and their dynamics. We created a set of more than 20 double mutants specifically labeled with a Förster resonance energy transfer (FRET) pair via the insertion of an unnatural amino acid and a single cysteine. Ensemble time correlated single photon counting (eTCSPC) revealed their corresponding population fractions and provides with structural information. Nevertheless, single molecule FRET, in confocal illumination, showed fluorescence lifetime averaging in timescales faster than diffusion time. To fully characterize the dynamics we used filtered fluorescence correlation spectroscopy which combined with eTCSPC represent a time resolution of seven orders of magnitude (ns to ms). In all, we used the measured distance network to generate a FRET restrained model of the three conformers with high precision. The open conformer appears readily available for substrate binding; the close conformer is very similar to the covalent enzyme-substrate adduct in the T26E mutant of T4L; the third conformer appears more compact than the adduct form which, at present, has not been reported in over more than 440 entries in the protein data bank.

1776-Pos Board B668

Photoblinking and Photobleaching of Single Molecule Fluorescent Probes Induced by Mn^{2+}

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Photophysical phenomena leading to blinking and irreversible photobleaching represent a major obstacle that limits the utility of organic fluorophores in fluorescence-based techniques involving the detection of small numbers of molecules. In recent work, we demonstrated that the coordination of paramagnetic transition metals (e.g. Mn^{2+}) to DNA induces intersystem-crossing in dyes covalently attached to the nucleic acid. This results in fluorescence quenching, triplet blinking and accelerated photobleaching. The increase in triplet formation in the presence of manganese was demonstrated using transient absorption techniques and fluorescence correlation spectroscopy for a series of rhodamine and cyanine dyes, including TAMRA, Cy3 and Cy5. These results are particularly relevant for single-molecule or fluorescence correlation spectroscopy experiments aimed to study enzymes that act on DNA, where Mn^{2+} is used to relax the sequence-specificity of enzymes that catalyze phosphoryl transfer reactions (e.g. polymerases and restriction endonucleases).

1777-Pos Board B669

One and Two Photon Fluorescence Correlation Spectroscopy on Proteins in Glucose Solutions

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Cryopreservation is a powerful technology with many applications in biomedical fields from organ preservation to cellular research. Simple sugars such as glucose and fructose are among the most widely used cryopreserving agents, yet despite their widespread use, the mechanism through which sugars protect